DEVELOPMENT AND CHARACTERIZATION OF A PROTOTYPE FOR NMR STUDIES OF MAMMALIAN CELLS

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FINAL REPORT

1. Summary

The overall goal of this research was to design, fabricate and evaluate a perfusion apparatus with a sample compartment yielding adequate sensitivity for nuclear magnetic resonance (NMR) studies of immobilized animal cells in horizontal 4.7 Tesla magnets. The system studied consisted of insulin-secreting recombinant mouse pituitary AtT-20 and mouse insulinoma βTC3 cells entrapped as single cells or spheroid aggregates in calcium alginate/poly-L-lysine (PLL)/alginate microbeads. This system realistically simulates immunoisolated implantable devices, which have exceptional potential for the long-term treatment of endocrine disorders, primarily diabetes mellitus. Our work during the three years of the project focused on engineering functional, insulin-secreting microbeads containing immunoisolated cells; on designing, fabricating and characterizing an NMR-compatible fixed bed reactor and support perfusion circuit which can maintain entrapped cells for prolonged periods and under various culture conditions; and on evaluating the bioenergetics and secretory function of entrapped AtT-20 spheroids on a long-term basis and of entrapped βTC3 cells under zero and saturating glucose concentrations. Preliminary investigations on the effect of hypoxia on bioenergetics and insulin secretion from entrapped βTC3 cells were also performed.

2. Entrapped cell systems

Our initial proposal included studies only with preparations of recombinant, insulin-secreting AtT-20 cells. This is an excellent model system for fundamental studies on regulated and constitutive secretion and on the effect of entrapment on cell function. However, AtT-20 cells cannot be used in the development of a bioartificial pancreas because they are not glucose responsive and they also secrete pituitary hormones. We thus extended our studies to a system consisting of microbeads containing immunoisolated βTC3 mouse insulinomas. The βTC3 cell line (kindly provided by Dr. Shimon Efrat, Albert Einstein College of Medicine, Bronx, New York) regulates proinsulin biosynthesis and insulin secretion by the level of glucose in the medium and retains glucose responsiveness for about 50 generations in culture [4]. Implantation in diabetic rats of βTC1 cells, an earlier variant of the βTC3 line with similar properties, restored

normoglycemia for more than a month [5]. Thus, βTC3 cells are promising for the development of an artificial pancreatic tissue that can be fabricated reproducibly at a medically relevant scale.

3. Characterization of immunoisolated cell preparations

An investigation was first carried out to characterize the stability of immunoisolated preparations of entrapped cells. Indeed, the cells used in our studies have a high growth potential, so it was necessary to examine if they grew in the alginate matrix, possibly rupturing the beads after prolonged cultivation. Even in the absence of bead rupture, growth of cells is undesirable since it results in the device secreting increased amounts of insulin. Increased insulin secretion may revert diabetes to hyperinsulinemia and hypoglycemia, a serious pathological condition.

Experiments were first conducted with AtT-20 cells which exhibit little contact inhibition. If growth of these cells could be suppressed without compromising function, the same could be true with more contact-inhibited cells, such as the βTC3 line. Preliminary experiments showed that entrapped AtT-20 cells behaved best when they were preaggregated into spheroids and entrapped as such in the calcium alginate gel. Freshly trypsinized cells entrapped without preaggregation in calcium alginate and cells or spheroids encapsulated in hollow beads obtained by solubilization of the calcium alginate core showed inferior behavior [8].

AtT-20 spheroids were prepared by inoculating trypsinized monolayers in spinner flasks. By controlling the culture initiation and propagation parameters, spheroids increased in size with time but maintained a narrow size distribution [8]. Spheroids of 450 μm average diameter were entrapped in calcium alginate/PLL/alginate beads of roughly 3 mm in diameter. The immobilization protocol used was similar to the procedure of Sun [12], except that the bead core was not solubilized after immobilization, so spheroids remained imbedded in the calcium alginate matrix. The cell loading was $3x10^7$ cells/ml biopolymer [8,10]. This density of AtT-20 cells results in the system dynamics being controlled by the rate of reaction; diffusion through the immobilization polymer is not limiting [14,15]. The MW cutoff imposed by the calcium alginate/PLL/alginate system was found to be between 45 and 66 kDa [15]. Figures 1 in [8] and 1 in [10] show a schematic and photomicrographs of the preparation.

The metabolic and basal secretory functions of entrapped AtT-20 spheroids were evaluated and compared to those of free spheroid controls in long-term fed-batch and perfusion spinner flask cultures [8,10]. In free spheroid cultures, the total cell number and spheroid size increased with time; the overall metabolism and basal secretion increased to a lesser extent. This was probably

caused by an increase in nutrient-limited and/or necrotic cells as spheroid size increased. In entrapped spheroid cultures, the total cell number, the spheroid size and the overall metabolism and basal secretion remained roughly constant over a month. The specific metabolic and secretory rates were initially the same for free and entrapped spheroids, indicating no significant damage during immobilization.

Immobilized βTC3 cells behaved in a similar way over a period of two weeks when they were entrapped as single cells or small spheroids in calcium alginate/PLL/alginate. Thus, experiments with this system were carried out with the simpler preparation, i.e., cells from freshly trypsinized monolayers. Cells were entrapped at densities of 3-7x10^7 cells/ml. Cell viability after each step of the immobilization procedure was evaluated by the trypan blue exclusion method. Viability decreased from ~97% in freshly trypsinized cells to ~85% in calcium alginate/PLL/alginate beads one day after addition of the PLL layer. This decrease is statistically significant but not detrimental to the usefulness of the preparation. A preliminary assessment of long-term viability was carried out by propagating entrapped βTC3 cells in a spinner flask fed roughly every two days to maintain the glucose concentration above 1 mg/ml. Viability was ~73% after 30 days in culture [9].

The responsiveness of entrapped βTC3 cells to changes in glucose levels was investigated next. Due to the hypersensitivity of βTC3 cells to glucose [4,13], the low glucose concentration used was 0 mM; the high glucose concentration was 25 mM. Secretion of insulin-related peptides (IRP) occurred at a rate above 60 μU/10^5 cells-hr in the high glucose medium, and at roughly 6 μU/10^5 cells-hr at zero glucose (Figure 3 in [9]). Thus, the preparation is glucose-responsive, at least for these two glucose levels.

The above results indicate that entrapment in calcium alginate/PLL/alginate suppresses cell growth and stabilizes the metabolic and secretory activities of the preparation. The exact cause of growth suppression was not investigated, but it appears to be due to a combination of physical (space restriction) and chemical (reduced concentration of macromolecular nutrients) effects. In any case, this type of behavior is most promising for the development of stable artificial tissues based on transformed cells.

4. Design of perfusion system

Figures 5 A and B in [10] show a schematic of the bioreactor and the perfusion circuit used in NMR experiments with entrapped AtT-20 spheroids and βTC3 cells. The bioreactor was

cylindrical with a volume of 22 ml. It consisted of a glass tube with two Teflon end pieces. The bulk of medium in the perfusion loop was in a jacketed, aerated spinner flask (medium reservoir). Medium was circulated through the packed bed by a peristaltic pump at 30 ml/min. The pressure drop across the bed was approximately 30 cm of water. Spent medium was replenished with fresh at various flow rates. The liquid volume in the reservoir was maintained constant by positioning the medium withdrawal tube at a fixed level above the bottom of the flask. Dissolved oxygen and temperature were monitored at various places in the perfusion loop by sensors in flow-through cells. The sensors were interfaced to a personal computer for continuous, on-line data acquisition, so that a "fingerprint" of the system operation was available at the end of each experiment.

The perfusion system is equivalent to entrapped cells being suspended in a well-stirred vessel containing the same volume of medium as the perfusion loop. The advantage of the perfusion configuration is that cells are segregated from the bulk of medium, so relatively high cell densities are achieved within the radiofrequency (RF) coil where NMR signal is collected from.

5. NMR spectroscopy and imaging

NMR data were obtained with a horizontal bore SISCO 200/33 spectrometer operating at 200.057 MHz for ^{1}H and 80.984 MHz for ^{31}P. The usable diameter of the bore was 12.5 cm, and the maximum gradient strength was 10 Gauss/cm. Data were acquired using a double-tuned $^{31}P/^{1}H$ home built probe based on the modifications proposed by Chang et al. [1] on the original design of Schnall et al [11]. The radiofrequency antenna consisted of a loop-gap resonator that surrounded the cylindrical shape of the bioreactor. Typical acquisition parameters for the ^{31}P experiments were: 3000 Hz spectral width, 4096 complex points per free induction decay (FID), 90° pulses, 3 seconds relaxation delay and 2048 transients. ^{1}H NMR images were acquired with a spin echo sequence using an echo time of 50 ms, a repetition time of 2 s, a field of view of 6 cm, and a matrix of 128x128 phase-encoding steps [2,9,10].

6. RF homogeneity and packing uniformity

RF homogeneity in the bed was investigated by obtaining ^{1}H NMR images both transverse and parallel to the reactor axis. Perfusion was stopped during acquisition to avoid motion artifacts. Uniform bright images, outlining the shape of the bioreactor and free of field artifacts, were

observed, demonstrating the uniformity of the coil design (Figures 1A and B in [2]).

To assess the packing uniformity of the bed, multiple slice ^{1}H NMR images of the reactor loaded with entrapped AtT-20 spheroids were obtained in directions both transverse and parallel to the reactor axis. Acquisition of these images under perfusion resulted in diminution of signal from the perfusate and demarcation of the alginate beads. Figure 6 in [10] shows a sequence of ^{1}H NMR images transverse to the reactor axis. The packing uniformity can be clearly seen. When the NMR data were processed so as to obtain phase-sensitive images, the phase changes formed parallel bars in the absence of medium flow (Figure 7B in [10]). In the presence of flow, the phase bars were distorted at locales of higher medium velocity, such as interbead spaces (Figure 7C in [10]). The phase-sensitive images show the absence of any gross flow channelling through the bed, indicating the prevalence of plug flow through the bioreactor. Such images were routinely obtained during the course of long-term experiments to ensure, along with visual observations, that the bed remained uniformly packed.

7. Differential operation of the bed

Under the operating conditions employed, dissolved oxygen (DO) is the only nutrient that can measurably decrease in a single-pass through the bed. By positioning DO probes in flow cells upstream and downstream from the reactor, it was found that the inlet DO concentration ranged from 40-75% air saturation, whereas the drop in DO across the bed was no more than 15% of the inlet value. Thus, both the inlet and outlet DO concentrations were well above the range where oxygen becomes limiting.

A more thorough evaluation of the differential operation of the reactor was performed by acquiring ^{31}P 1-Dimensional Chemical Shift Imaging (1D-CSI) spectra of entrapped AtT-20 spheroids in directions transverse and parallel to the axis of the bed. Figure 3 in [2] shows ^{31}P NMR spectra acquired from slices orthogonal to the bioreactor axis. Similar spectra were also acquired from slices parallel to the bioreactor axis. Data from both experiments were analyzed by integrating the metabolite resonances in each spectrum, and calculating the following metabolite ratios: PCr/P_i, P_i/β-NTP, and PCr/β-NTP. The PME/PDE ratio was not calculated because the low signal-to-noise ratio of the spectra and the broad linewidth of the P_i resonance did not allow for an accurate measure of these metabolites. No statistical differences were observed among the metabolite ratios in the various slices in both orientations (Table 1 in [2]). These experiments

demonstrate macroscopic homogeneity of cellular bioenergetics in the reactor and differential operation of the bed.

8. Long-term study of immunoisolated AtT-20 spheroids

Metabolic, secretory and bioenergetic indicators were followed on a long-term basis for immunoisolated AtT-20 spheroids. Concentrations of glucose and IRP were determined periodically in samples from the medium reservoir in the perfusion circuit. Figures 6A and B in [2] show the glucose and IRP concentration profiles, as well as the medium replenishment flow rate during the course of the experiment. Concentrations changed because of changes in the replenishment flow rate and/or because of changes in the metabolic and secretory activities of the culture. To differentiate between these two causes, concentration profiles were also simulated by solving the mass balance equations for glucose and IRP and by assuming a Monod-type expression for glucose uptake and a constant specific IRP secretion rate. The simulation results are shown by the solid lines in Figures 6A and B in [2]. The model closely tracks the glucose and IRP data during the first 45 days of the experiment, but it deviates significantly from them thereafter. The increase in glucose and decrease in IRP concentrations measured experimentally during the later part of the experiment were apparently caused by cell death.

^{31}P NMR spectra of entrapped AtT-20 spheroids were collected continuously for a period of 70 days from the same cell preparation. Figure 4 in [2] shows a stack of representative spectra acquired during this experiment. Ratios of detectable metabolites were determined, and several temporal changes were observed. Specifically, the ratios P_i/β-NTP and PCr/β-NTP changed slightly during the first 40 days, and then increased considerably (Figures 5A and 5C in [2]); the ratio of PCr/P_i (Figure 5B in [2]) exhibited an almost linear decrease with time. The PME/PDE ratio exhibited what appeared to be a three-phased response with a sharp decrease between days 14 and 20, followed by a slower decrease up to day 35, and then stabilizing until the end of the experiment (Figure 5D in [2]). Values of this ratio were not determined for the first 14 days, since PDE resonances were not detected during that period (Figure 4 [2]). The observed decrease of this ratio between days 10 and 30 was attributed to the continuing increase of the PDE metabolites. The ratio of the PME components, PC/PE, did not appear to exhibit a significant change during the experiment with the exception of the last three data points (Figure 5E in [2]). The higher values of these points are not significant due to the higher error in the calculation of the PE component of this ratio, and the overall lower signal-to-noise ratio of all metabolites during the last few days of

the experiment (Figure 4 in [2]). Finally the ratio of the PDE components, GPC/GPE, increased progressively from the day PDE resonances appeared (Figure 5F in [2]).

9. Studies with immunoisolated βTC3 cells at various glucose concentrations

Results reported in the literature indicate significant changes in islet cell bioenergetics, assessed by the ATP/ADP ratio, with glucose concentration in the surrounding medium [6,7]. To evaluate the bioenergetic changes of entrapped βTC3 cells induced by changes in glucose levels, ^{31}P NMR spectra were collected from cell-loaded microbeads perfused with medium containing 0 or 16 mM glucose. Beads were first perfused with glucose-containing medium for approximately 16-20 hours, then were switched to glucose-free medium for 4 hours, and the procedure was repeated three more times with a particular cell preparation. All media contained glutamine at approximately 6 mM. A total of five experiments of this type have been carried out with five different βTC3 cell preparations. Figure 1 shows a set of typical results on β-NTP levels and IRP secretion at the two glucose concentrations. Results from all experiments agree that IRP secretion at 0 mM glucose occurred at a much lower rate than at 16 mM, but the β-NTP level at 0 mM was not substantially different over the four-hour period from the β-NTP level at 16 mM. We are currently investigating possible differences in the levels of other cellular metabolites at the two glucose concentrations.

10. Preliminary studies with immunoisolated βTC3 cells under hypoxia

Results reported in the literature demonstrate a strong dependence of islet cell bioenergetics and insulin secretion on the level of DO in the surrounding medium [3,7]. Due to their transformed phenotype, βTC3 cells may be less susceptible to hypoxia, which would be a significant trait in tissue engineering applications involving these cells. Cells will experience hypoxic conditions after implantation due to the absence of vascularization and the diffusional limitations imposed by the alginate matrix.

To carry out NMR and secretion studies at various DO levels, the perfusion system was modified by the introduction of a deoxygenator consisting of gas permeable silicone tubing in a glass shell. Medium flowed in the tubing and air or pure nitrogen was supplied in the shell. Figures 2 and 3 show typical sets of data obtained by switching from a high to a low DO level, as measured with a DO probe positioned approximately 12 feet from the bed. The β-NTP level appeared to exhibit a decrease as DO was lowered, whereas the rate of IRP secretion did not

change. We are in the process of repeating this type of experiment at various DO levels to better evaluate the bioenergetic and secretory changes exhibited by entrapped cells. In any case, the type of secretory behavior indicated in Figures 2 and 3 is promising for the development of an artificial pancreatic tissue based on βTC3 cells.

11. Conclusions

We have developed stable preparations of immunoisolated AtT-20 spheroids and βTC3 cells in calcium alginate/PLL/alginate beads. We have designed, fabricated and characterized a fixed-bed perfusion bioreactor and support circuit which are compatible with a 4.7 T, horizontal-bore NMR spectrometer and can maintain the entrapped cells in the long-term and under various culture conditions. This system allows the acquisition of ^{31}P NMR spectra at cell densities typical of tissue engineering applications. The RF field in the loop-gap resonator was uniform and cellular bioenergetics were the same at all places in the bioreactor. Levels of high-energy metabolites in immunoisolated AtT-20 spheroids remained roughly constant for 45 days under continuous perfusion, after which they rapidly declined as cell died. Preparations of entrapped βTC3 cells secreted at a much lower rate at 0 versus 16 mM glucose, but NTP levels did not differ substantially at the two glucose concentrations when the zero glucose level was kept for up to 4 hours. Reduced DO levels hand appeared to cause a decrease in NTP but no significant change in the rate of secretion. Aspects of βTC3 cell behavior indicate that an artificial pancreatic tissue based on these cells may be superior to one based on normal islets.

12. References

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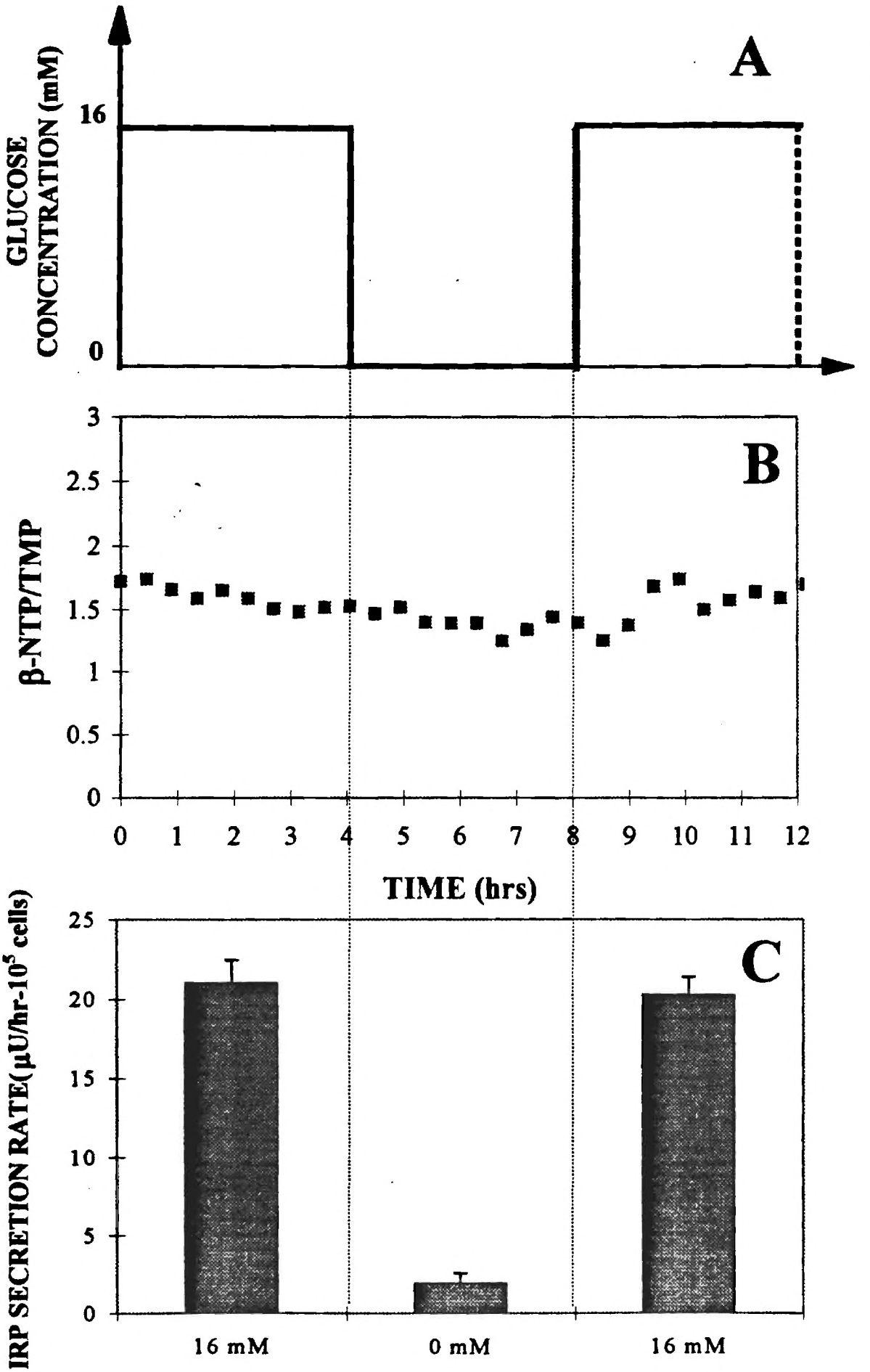
13. Figure captions

Figure 1 β-NTP levels and rates of IRP secretion from entrapped βTC3 cells perfused with media containing 0 or 16 mM glucose. Calcium alginate/PLL/alginate beads of 1 mm diameter containing $7x10^7$ cells/ml alginate were perfused with glucose-containing medium for 16-20 hours, then switched to glucose-free medium for 4 hours, and the sequence was repeated three more times. TMP stands for tetramethylphosphate, a certain amount of which in a capillary tube in the fixed bed served to normalize the metabolite resonances. The Figure shows the β-NTP/TMP ratio and the IRP secretion rates during a zero glucose episode and during the four hours before and after the episode when beads were perfused with 16 mM glucose.

Figure 2 β-NTP levels and rates of IRP secretion from entrapped βTC3 cells perfused with media containing different DO levels. Calcium alginate/PLL/alginate beads of 1 mm diameter containing $7x10^7$ cells/ml alginate were perfused with aerated, glucose-containing medium for 16-20 hours, then switched to glucose-containing medium with a reduced DO level for 5 hours. The reported DO levels were measured with a probe in a flow-through cell positioned approximately 12 ft from the bioreactor.

Figure 3 Same as Figure 2, except that entrapped βTC3 cells were perfused with glucose-containing medium with a reduced DO level for a longer period of time.


A
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0 1 2 3 4 5 6 7 8 9 10 11 12
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16 mM
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16 mM

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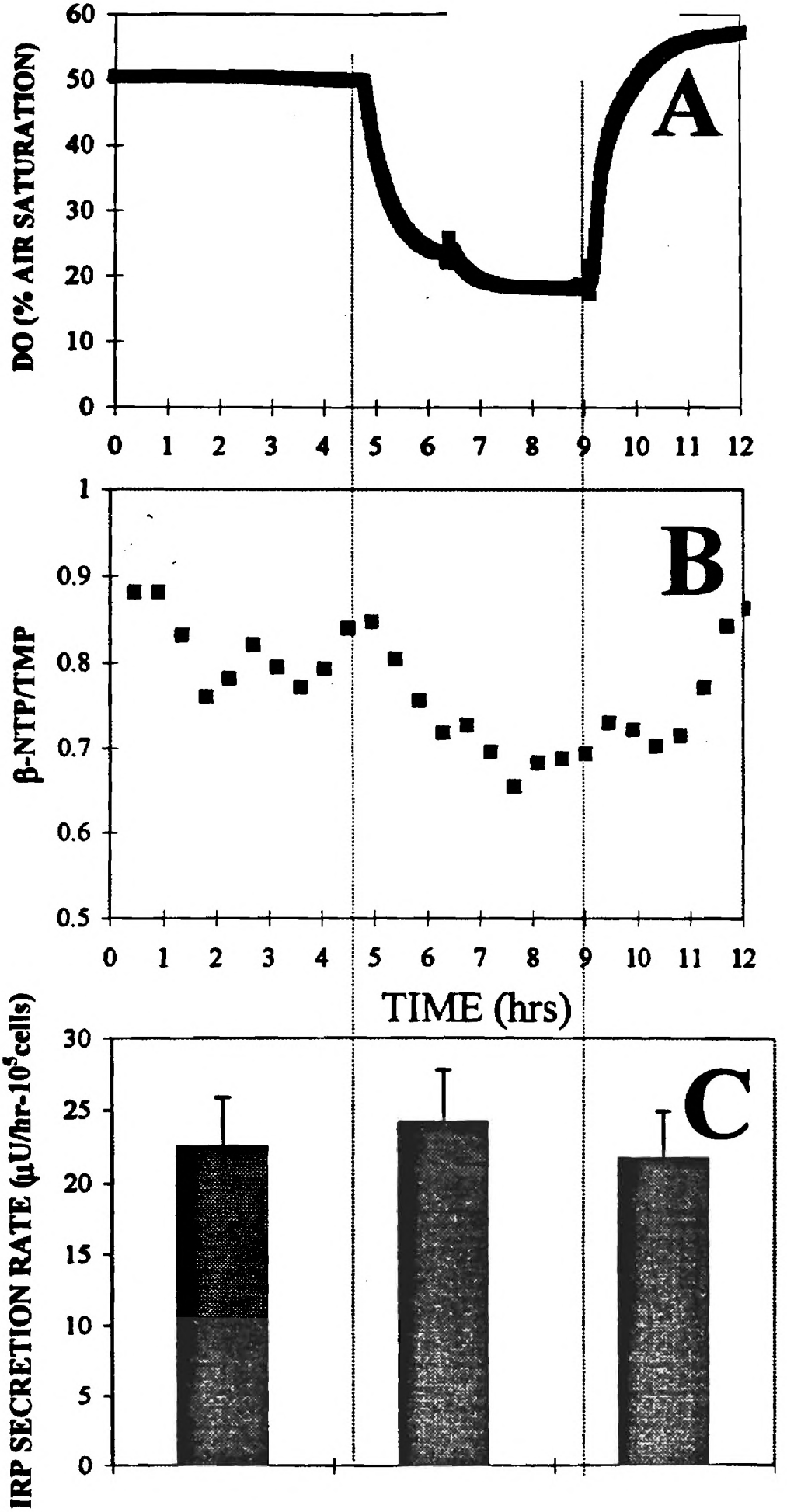
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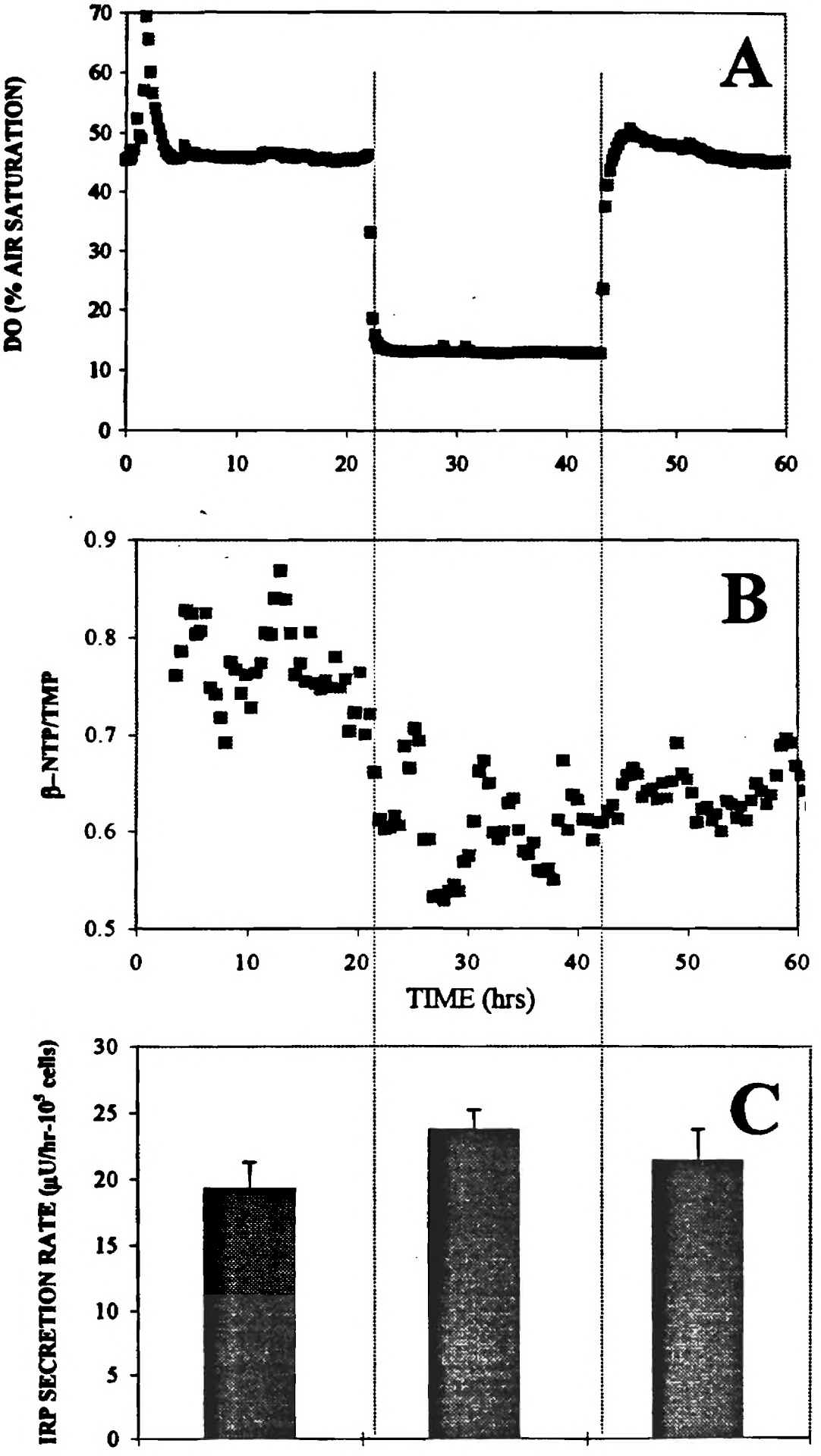
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IRP SECRETION RATE (μU/hr-10⁵ cells)
30
25
20
15
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14. Publications based on research supported by the Whitaker grant

Papas, K.K.; Constantinidis, I.; Sambanis, A. Cultivation of recombinant, insulin-secreting AtT-20 cells as free and entrapped spheroids", *Cytotechnol.*, **13**: 1-12 (1993).

Tziampazis, E.; Sambanis, A. Tissue engineering a bioartificial pancreas: Modeling the cell environment and device function. *Biotechnol Progr.*, in press (1994).

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Sambanis, A.; Papas, K.K.; Constantinidis, I. Towards the development of artificial endocrine tissues: I. Stability and function of immunoisolated, transformed insulin-secreting cells. Submitted to *Biotechnol. Bioeng.*

Constantinidis, I.; Long, R.C. Jr.; Erickson, M.; Sambanis, A. "Towards the development of artificial endocrine tissues: II. ^{31}P NMR spectroscopic studies of entrapped insulin-secreting cells". Submitted to *Biotechnol. Bioeng.*

15. Conference presentations based on research supported by the Whitaker grant

Invited Presentations

A. Sambanis, "Tissue Engineering Bioartificial Endocrine Organs: The Case of Diabetes Mellitus," U.S.-U.K. N+N Cellular Engineering Workshop, Chester, England, September 15-17, 1993.

A. Sambanis and I. Constantinidis, "Tissue Engineering a Bioartificial Endocrine Pancreas," Keystone Symposium on Tissue Engineering, Taos, New Mexico, February 20-26, 1994.

A. Sambanis, "On-Line Monitoring of Artificial Tissues by NMR", Engineering Foundation Conference on Cell Culture Engineering IV, San Diego, California, March 7-12, 1994.

Other presentations

Sambanis, A., Papas, K.K., Constantinidis, I., Long, R.C., Jr. and Dixon, W.T., "Development and characterization of a prototype for NMR studies of mammalian cells." The Whitaker Foundation Conference, Snowbird, UT, August 1992.

Papas, K.K. and Sambanis, A., "Characterization of the Metabolic and Secretory Behavior of Free and Immobilized Insulin-Producing AtT-20 Spheroids." Annual American Institute of Chemical Engineers Meeting, Miami Beach, Florida, November 1-6, 1992.

Constantinidis, I., Sambanis, A. and Long, R.C., Jr., "NMR Studies of Perfused, Immunoprotected, Insulin-Secreting Endocrine Cells on a 4.7 T Horizontal Magnet." Society of

Magnetic Resonance Imaging 11th Annual Meeting, San Francisco, California, March 27-31, 1993.

Sambanis, A., Papas, K.K., Constantinidis, I., Long, R.C., Jr. and Dixon, W.T., "Development and characterization of a prototype for NMR studies of mammalian cells." The Whitaker Foundation Conference, Snowbird, UT, July 30 - August 1, 1993.

Constantinidis, I., Sambanis, A. and Long, R.C., Jr., "Long-Term NMR Studies of Perfused Immunoisolated Endocrine Cells." Society of Magnetic Resonance in Medicine 12th Annual Meeting, New York, New York, August 14-20, 1993.

Sambanis, A., Papas, K.K. and Constantinidis, I., "Tissue Engineering of Endocrine Animal Cells," 1st International Conference on Cellular Engineering, Stoke-on-Trent, England, September 12-15, 1993.

Sambanis, A., Papas, K.K., Constantinidis, I. and Long, R.C., Jr., "Towards the Development of a Bioartificial Pancreas: Fabrication and Non-Invasive Monitoring of Microbeads Containing Insulin-Secreting, Transformed Cells," Annual American Institute of Chemical Engineers Meeting, St. Louis, Missouri, November 7-12, 1993.

Tziampazis, E. and Sambanis, A., "Modeling the secretory response of entrapped endocrine cell preparations," Annual American Institute of Chemical Engineers Meeting, St. Louis, Missouri, November 7-12, 1993.

Sambanis, A., "Development of Artificial Endocrine Tissues", Institute of Bioengineering and Bioscience Workshop, Georgia Institute of Technology, Atlanta GA, November 20, 1993.

Sambanis, A., "Endocrine Cells in Bioprocessing and Tissue Engineering: Potential and Limitations", University System Symposium, Georgia State University, Atlanta, Georgia, May 6-7, 1994.

Tziampazis, E. and Sambanis, A., "Insulin Secretion in a Non-Vascular Bioartificial Pancreas", University System Symposium, Georgia State University, Atlanta, Georgia, May 6-7, 1994.

Constantinidis, I., Flanders, P.C., Papas, K.K., Kang, H., Long, R.C. Jr. and Sambanis, A., "Correlations of NMR Data to Secretion of Insulin in Perfused Immunoprotected Endocrine Cells", Society of Magnetic Resonance Annual Meeting, San Francisco, California, August 6-12, 1994.

16. Grant support for continuation of research in this area

Tissue Engineering of Endocrine Cells: Development of Implantable Bioartificial Organs
Funded by the National Science Foundation at $231,000 for the period 8/15/94-7/30/97.

Protein Production by Animal Cells in Culture
Funded by the Georgia Research Alliance at $150,000 for the period 7/1/94-6/31/95; $107,000 for Georgia Tech and $43,000 for Emory University.